Research Article

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Importance of Connexin-43 based gap junction in cirrhosis and acute-on-chronic liver failure

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Background & Aims: In cirrhosis, superimposed inflammation often culminates in acute-on-chronic liver failure (ACLF) but the mechanism underlying this increased sensitivity is not clear. Cx43 is a ubiquitous gap junction protein that allows transmission of signals between cells at a much higher rate than the constitutively expressed gap junctions. The aims of the study were to test the hypothesis that inflammation drives the increased expression of hepatic Cx43 and to determine its role by Cx43 inhibition.

Methods: Four weeks after bile-duct ligation (BDL) or sham operation, rats were treated with an anti-TNF antibody, or saline; with or without LPS (1 mg/kg); given 3 h prior to termination. Biochemistry and cytokines were measured in the plasma and hepatic protein expression (NFkB, TNFα, iNOS, 4HNE, Cx26, 32, and 43) and confocal microscopy (Cx26, 32, and 43) were performed. The effect of a Cx43-specific inhibitory peptide was studied in a mouse BDL model.

Results: BDL animals administered LPS developed typical features of ACLF but animals administered infliximab were relatively protected. Cx26/32 expression was significantly decreased in BDL animals while Cx43 was significantly increased and increased further following LPS. Infliximab treatment prevented this increase. However, inhibiting Cx43 in BDL mice produced detrimental effects with markedly greater hepatocellular necrosis.

Conclusions: The results of this study show for the first time an increased expression of hepatic Cx43 in cirrhosis and ACLF, which was related to the severity of inflammation. This increased Cx43 expression is likely to be an adaptive protective response of the liver to allow better cell-to-cell communication.

Keywords: ACLF; Connexin; Gap junction; Cytokine; Infliximab; Inflammation.
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Abbreviations: Cx, Connexin; ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; iNOS, inducible nitric oxide synthase; GJIC, gap junction intercellular communication; HNE, hydroxyl non-enol; NFkB, nuclear factor kB.

Introduction

Acute-on-chronic liver failure (ACLF) is a newly described entity that is used to indicate a group of patients with previously well-compensated cirrhosis that have an increased risk of multiorgan failure and death, following a precipitating event such as infection. The mechanism why a patient with cirrhosis is susceptible to rapid deterioration, with acute worsening liver function following a superimposed inflammatory insult is unclear.

Hepatocytes communicate with their immediate neighbour through cytosolic exchange of small molecules (<1 kDa) such as Ca^{2+}, NAD^{+}, glutamate, ATP, prostaglandin E2, and glutathione, through intercellular gap junction (GJ) channels [1,2], which are primarily composed of two connexon hemichannels. Docking of connexons (Cxz) from adjacent cells forms membrane pores, which play an important role in maintaining differentiated hepatic functions [3]. The wide distribution and conservation of Cxs in different cells and organisms, as well as their modulation at both transcriptional and translational levels, indicate their fundamental importance for hepatic cell function [4,5]. Liver GJ are comprised mainly of Cx32 and Cx26, which serve as minor GJ proteins, and they are primarily located within the hepatocyte plasma membrane [3,6]. Cx43 is a ubiquitous GJ protein that allows transmission of signals between cells at a much higher rate than the constitutively expressed GJ Cx26 and Cx32, which is increased during hepatic inflammation [1,7,8]. In cirrhosis, overproduction of the pro-inflammatory cytokine TNFα is involved in the progression of liver injury. Infliximab, a chimeric anti-TNF antibody, has been shown to reduce inflammation in several clinical situations [9,10].

The aim of the study was to test the hypothesis that inflammation drives the increased expression of hepatic Cx43 and to determine its role by Cx43 inhibition using specific inhibitory peptides.

Materials and methods

Animals

All animal experiments were conducted according to the Home Office guidelines under the UK Animals in Scientific Procedures Act 1986. Male Sprague-Dawley

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(SD) rats, weighing 220–250 g and male C57BL/6 mice (weighing 25–30 g) were used (Charles-River Laboratories UK Ltd.). Housing conditions were as detailed previously [11].

Bile-duct ligation (BDL)

BDL or a sham operation was performed as described previously [11]. In the first experiment, four weeks after surgery, BDL rats were administered infliximab (10 mg/kg; intraperitoneal (IP) injection) or saline for three consecutive days and were randomised 3 h prior to sacrifice, to receive either lipopolysaccharide (LPS; 1 mg/kg, IP) or saline. The final study groups were: (1) Sham + saline (n = 10), (2) Sham + LPS (n = 6), (3) BDL + saline (n = 12), (4) BDL + infliximab (n = 7), (5) BDL + LPS (n = 8) and (6) BDL + infliximab + LPS (n = 6).

Due to the nature of the available reagents, the second experiment was performed in mice. BDL surgery was performed in a similar manner and the experiments terminated after 14 days; the final study groups were (1) Sham + saline (n = 4), (2) BDL + saline (n = 6), (3) BDL + LPS (n = 6). At the end of the experimental period, all animals in the different study groups were sacrificed; blood and snap frozen liver tissue were immediately processed and stored at −80 °C until analysis was performed.

Anti-Cx43 peptide treatment in a mouse model of BDL

Additional mice (n = 20) underwent BDL procedure (plus 3 sham-operated controls). In addition, in all of the BDL animals, an osmotic pump (Azlet, Charles-River UK) was placed subcutaneously behind the neck at the time of surgery. BDL animals were divided into two groups (n = 10 in each), those receiving either a continuous infusion (2 mM at 0.25 μl/h via the osmotic pump) of a peptide previously demonstrated to inhibit Cx43 (SRPTEKTIFII, Protein Peptide Research (PPR), Fareham UK) or a control scramble peptide (ITTPSIFKEIR, PPR UK) [12,13]. After 14 days, liver tissue samples were collected from the animals under terminal anaesthesia. Liver tissue was processed for H&E staining and confocal microscopy. The number of Cx43 positive dots was counted and expressed as dots/1000 μm area.

Measurement of plasma biochemistry and cytokines

Biochemical parameters were analyzed by Cobas (Roche-diagnostics, Burgess Hill, West Sussex, UK) and for TNFα cytokine, the rat inflammation cytometric bead array (CBA, Becton Dickenson (BD), UK) kit was used, measured by flow cytometry FACS Canto™ II flow cytometry system (BD™ Sciences) (CANTO II, BD, UK).

Immunohistochemistry and histological damage analysis

Tissue processing: Paraformaldehyde (PF) fixed mouse liver specimens were removed and stored at 4 °C in PBS containing 30% sucrose for cryoprotection. H&E staining was performed and immunohistochemistry was carried out using appropriate antibodies (Supplementary data).

Immunofluorescence analysis

For immunofluorescence analysis, frozen mouse liver sections were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe B.V., The Netherlands), and sectioned with a cryostat (~12 μm in thickness). Staining was performed as described in Supplementary data.

Western blot analysis

Proteins were isolated from fresh-frozen liver tissues, which were homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.6) (with a protease inhibitor cocktail (Sigma-Aldrich Inc., UK), protein methyl sulphonyl fluoride (PMSF in ethanol) and ethylene diamine tetra acetic acid (EDTA)), using a tissue homogenizer. Cx26, 32, and 43, NFkBP65, TNFα, inducible nitric oxide synthase (iNOS) and mouse anti 4-hydroxynonenol (HNE) protein expression was measured (Supplementary data).

Statistics

All data are reported as mean ± SEM. Six groups were analyzed by ANOVA if the *p*-value was significant, *post hoc* comparisons were done using the Newman-Kuels multiple range test and Student *t*-test or Mann-Whitney comparisons tests, as indicated in figure legends; *p* values less than 0.05 were considered significant. The software used included Microsoft Excel 2010 and GraphPad Prism 5.0.

Results

A tendency to decrease the body weight following surgery was observed in BDL rats, which stabilized and then increased at a similar rate to control animals, in the following weeks, throughout the study period. There were no statistically significant differences of body weight between the sham and experimental groups studied (data not shown).

Infliximab treatment attenuates plasma ALT and ammonia and restores albumin levels of BDL rats challenged with or without LPS

Compared to sham-operated control, the levels of plasma ALT, bilirubin, and ammonia were significantly increased in BDL rats (p <0.0001, p <0.0001, and p <0.001, respectively; Table 1). Following LPS induction in BDL cirrhotic rats, the concentration of ALT was significantly further (p <0.05) elevated as compared to BDL alone. Infliximab treatment in BDL rats (±LPS) showed a significant reduction of ALT and ammonia levels compared to diseased rats (p <0.05, respectively). Moreover, albumin concentration was significantly higher in sham rats compared to BDL, with or without LPS challenge (p <0.0001, respectively). Infliximab treatment in BDL rats showed a significant increase of plasma albumin concentration (p <0.05).

Effect of infliximab on histological damage of control and experimental rats

Hepatic lobule from the sham rat (Fig. 1A) and sham rat that received LPS (Fig. 1B) revealed there was neither ductular epithelial cell proliferation nor inflammatory cell infiltration, whereas the BDL rat liver showed increased ductular epithelial cell proliferation and peri-ductular fibrosis, together with infiltration of inflammatory cells (Fig. 1C). Furthermore BDL + LPS rat liver (Fig. 1E) showed similar histology to BDL, and acute infliximab treatment did not alter liver histology in either BDL (Fig. 1D) and BDL + LPS rats (Fig. 1F).

Infliximab treatment ameliorates plasma and hepatic TNFα of BDL rats challenged with or without LPS

Compared to the sham-operated control, BDL rats challenged with or without LPS showed significantly higher plasma TNFα concentration (p <0.01 and p <0.01, respectively). Infliximab treatment in BDL rats (±LPS) significantly attenuated plasma TNFα concentration (p <0.05 and p <0.01, respectively) (Table 1). Furthermore, Western immunoblot analysis revealed hepatic TNFα protein expression (Fig. 2B) to be significantly increased in BDL rats (p <0.0001), which was further aggravated following LPS injection (p <0.0001). Infliximab treatment resulted in a significant (p <0.05) decrease in TNFα protein expression in BDL and BDL + LPS rats.

Infliximab treatment attenuates NFkB, iNOS, and 4-HNE protein expressions of BDL rats challenged with or without LPS

Compared to sham-operated control rats, protein expression of NFkB (Fig. 2A), iNOS (Fig. 2C), and 4-HNE (Fig. 2D) was significantly increased in BDL rats (p <0.0001, p <0.0001, and p <0.05, respectively). LPS administration to BDL rats showed a further significant increase of iNOS protein expression (p <0.0001) while